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Minireview

New Insights into the Interaction of Ras with the Plasma Membrane

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Ten years ago a series of papers showed that a complex set of signals are required for localization of Ras proteins at the plasma membrane. In this issue of *Cell*, Philips and coworkers shed new light on this problem by demonstrating that intracellular membranes and vesicular transport are involved in the passage of Ras proteins to the inner surface of the plasma membrane (Choy et al., 1999). The signals required for Ras proteins to be localized to the plasma membrane consist of two components. The first component comprises farnesylation of the cysteine of the C-terminal CAAX motif. This is followed by proteolytic removal of the AAX residues and finally carboxyl methylation of the C terminus. Variations of this pathway have now been found to act on many proteins, resulting in a wide range of related C-terminal modifications (reviewed by Glomset and Farnsworth, 1994). The CAAX modifications make the C terminus of Ras hydrophobic, the dominant contribution being made by the 15-carbon farnesyl prenoid group. However, the hydophobicity provided by the CAAX modification does not lead to stable plasma membrane binding of Ras, nor does it account for the selective localization of Ras to the plasma membrane that is observed. The consensus view that has developed is that a cooperative effect of the CAAX modifications and a second signal are required for plasma membrane targeting.

That second component of the membrane localization signals has been shown to be either S-acylation (palmitoylation) of cysteine residues in N- and H-Ras proteins or, in the case of the K-Ras4B protein, a polybasic stretch of amino acids. Both types of second signal occur in the 20–amino acid so-called hypervariable domain of the four mammalian Ras proteins (H-Ras, N-Ras, K-Ras4A, K-Ras4B) that separates the highly conserved body of the molecule, containing its nucleotide-binding and functional domains, from the CAAX motif. The long chain of the S-acyl group provides a highly hydrophobic substituent that is capable of conferring tight membrane binding. Stable targeting to the plasma membrane of N- and H-Ras may be achieved by the selective attachment of S-acyl groups when they contact that membrane in a random manner, resulting in a trapping mechanism. CAAX modifications facilitate this by providing an initial weak membrane-binding signal that allows access to the S-acylation machinery, the nature of which is still a matter of hot debate (Dunphy and Linder, 1998). On the other hand, the polybasic sequence of K-Ras4B is believed to interact electrostatically with the head groups of anionic phospholipids that are present in the cytoplasmic leaflet of the plasma membrane, which can account for the specific localization of K-Ras4B. The combination of these two signals is sufficient to target heterologous cytosolic proteins to the plasma membrane. Addition of Ras membrane localization signals has become a popular tool to generate constitutively activated versions of signaling molecules, such as kinases and guanine nucleotide exchange factors, whose activation is believed to be triggered by plasma membrane recruitment. This concept of a two-signal mechanism for subcellular localization also applies to other groups of lipid-modified proteins, such as those subject to N-terminal myristoylation, many of which also have nearby S-acyl groups or polybasic regions (reviewed in Resh, 1996). The differences in the mode of membrane binding with the two alternative second signals raises the possibility that these different anchoring modes may subtly alter the localization of Ras isoforms within the plane of the membrane, with functional consequences.

Plasma membrane localization of Ras is crucial for its function. This is where Ras interacts with its upstream activators, such as the Grb2-Sos nucleotide exchange factor complex that causes normal Ras to switch to its active GTP-bound state following activation of transmembrane receptors. This is also where active Ras recruits its targets—such as serine/threonine kinases of the Raf family, which trigger the ERK/MAP kinase pathway; phosphoinositide 3-kinase, which activates signaling molecules with PH domains; and the RalGEFs, which activate the Ral small GTPases.

The mechanism by which Ras proteins or other CAAX-modified proteins get to the plasma membrane is not fully understood. Philips and coworkers now show that Ras proteins don't directly travel to the plasma membrane but interact with intracellular membranes (Choy et al., 1999). Farnesylated Ras proteins first associate with the endoplasmic reticulum and then with the Golgi (see Figure 1). Intracellular Ras has been seen before, but the significance of this pool has taken a back seat compared to that at the plasma membrane. Interestingly, Choy et al. show that while N- and H-Ras seem to piggyback on exocytic transport vesicles following association with the endoplasmic reticulum and Golgi complex, K-Ras4B takes a faster route that may not involve the Golgi. Inhibition of vesicular transport with Brefeldin A blocks the transit of N-Ras to the plasma membrane demonstrating the importance of vesicular transport for Ras function. Experiments with chimeric GFP molecules showed that all the targeting information is contained in the hypervariable domains of the respective Ras proteins. Choy et al. report a surprisingly large amount of Ras associated with endomembranes and the cytoplasm even at steady state and show that transit of Ras to the plasma membrane takes several hours. These observation are somewhat at odds with a large

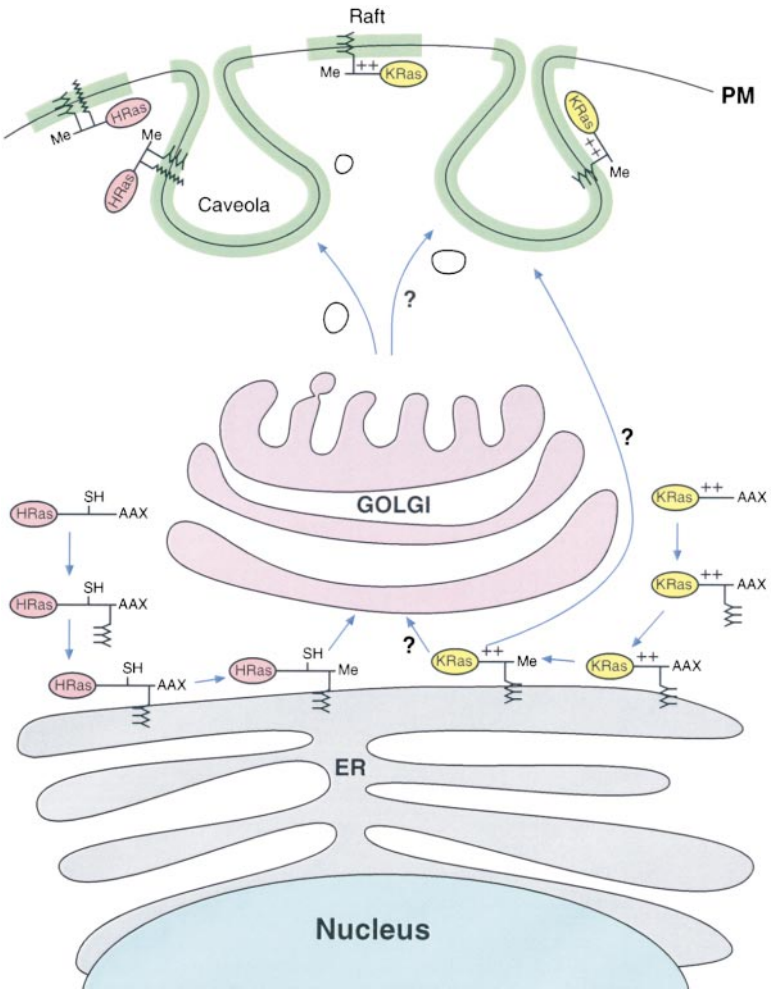

Figure 1. Ras Trafficking to the Plasma Membrane

Ras proteins are made in the cytoplasm and farnesylated (ww), then bind to the ER and are AAX proteolyzed and carboxyl methylated (Me). They then traffic to the plasma membrane via the secretory pathway (H-Ras) or perhaps through the cytoplasm (K-Ras). S-acylation (wwww) of H-Ras and the association of S-acylated H-Ras with lipid rafts and caveolae are depicted at the plasma membrane; however, another possibility is that S-acylation may occur on endomembranes. K-Ras association with the plasma membrane is assisted by its polybasic region (++).

body of earlier work. However, the endomembrane association of Ras may have been missed in previous studies (Hancock et al., 1991a) because the cytoplasmic staining that was interpreted as cytosolic rather than endomembrane was only observed for Ras mutants with defective second signals. In addition, fractionation of broken cells may displace weakly bound Ras from intracellular membranes, especially if it lacks S-acylation, thus causing it to be released into the cytosolic fraction.

What all three Ras proteins have in common is that the initial membrane interaction of their farnesylated forms occurs with the endoplasmic reticulum (ER). In both yeast and mammals this is the home of the multispanning enzymes known to catalyze proteolytic trimming of the AAX residues and carboxyl methylation of the resulting C terminus (Dai et al., 1998; Romano et al., 1998; Schmidt et al., 1998). It is even possible that the AAX protease and carboxyl-methyl transferase are physically associated in the membrane, which might facilitate their concerted action by substrate channeling. Previous work has shown that AAX proteolysis and carboxyl methylation may be required for efficient membrane binding of K-Ras4B (Hancock et al., 1991b). Choy et al. now provide evidence that newly synthesized farnesylated Ras proteins and carboxyl-methyl transferase are localized to the same cellular compartment. Furthermore, inhibition of carboxyl-methyl transferase with N-acetyl-S-*trans*-farnesylcysteine mislocalizes Ras to the cytoplasm, supporting a role for carboxyl methylation, and by extrapolation AAX proteolysis, in membrane association. It has been shown by a number of investigators that carboxyl methylation, which eliminates the negative charge on the C-terminal carboxylate group, has more effect on membrane binding of farnesylated proteins for which membrane affinity is borderline, than for geranylgeranylated proteins (Rando, 1996). Since carboxyl methylation is required for efficient plasma membrane binding, it is surprising that Ras-dependent signaling does not seem to be greatly affected when carboxyl methylation and AAX proteolysis are disrupted. In the mouse, homozygous deletion of *Rce1*, which encodes a major AAX protease, results in a large proportion of Ras being localized to the cytoplasm at steady state (Kim et al., 1999), but some embryos survive to term although they die in the first week after birth. Furthermore, fibroblasts cultured from knockout embryos grow as well as wild type in culture. This suggests that the key event for Ras signaling is farnesylation rather than the other posttranslational modifications.

In addition to Ras, other lipid-modified proteins have recently been shown to traffic to the plasma membrane via the endomembrane system. The Src family tyrosine

kinase Lck, which is N-terminally myristoylated and doubly S-acylated, associates with intracellular membranes and moves to the plasma membrane through the secretory pathway associated with its cell surface coreceptor CD4 (Bijlmakers and Marsh, 1999). However, Lck can still reach the plasma membrane even in the absence of CD4, and the N-terminal lipid modifications clearly specify intrinsic plasma membrane affinity. The related kinase Fyn binds to cellular membranes more quickly after synthesis than does Lck and may associate directly with the plasma membrane (van't Hof and Resh, 1997). The divergent behavior of these two very similar proteins may be due to subtle differences in their N-terminal regions. The spacing of the two S-acyl groups differs between Lck and Fyn, and the former has a predominance of negatively charged amino acids in the surrounding region whereas the latter is predominantly positively charged (Resh, 1996).

The mammalian Ras proteins are almost identical throughout most of their length and diverge only in the 20–amino acid hypervariable domain upstream of the CAAX motif, and in most assays the Ras isoforms are indistinguishable. This has led to the speculation that they are redundant. Recently, however, evidence for differential activities of the Ras isoforms has started to accumulate. Deletion of H-Ras or N-Ras has no apparent effect on mouse development although deletion of K-Ras is an embryonic lethal phenotype (Johnson et al., 1997). The studies with knockout mice suggest that the Ras proteins have some overlapping functions. However, if the K-Ras knockout lethality is not the consequence of some tissues only expressing K-Ras, then they also suggest that K-Ras performs a specific function that cannot be performed by H-Ras or N-Ras. The identity of this specific function is not clear although there are now indications that K-Ras4B is a better stimulator of Raf1 activity than H-Ras whereas H-Ras is a stronger stimulator of phosphoinositide 3-kinase activity (Yan et al., 1998). The work of Choy et al. reported in this issue adds another piece to this puzzle with the demonstration that Ras isoforms traffic by different routes to their site of action at the plasma membrane.

What about differential properties of Ras isoforms at the inner face of the plasma membrane where they are functionally active? Many studies have reported association of Ras proteins with plasma membrane domains enriched in cholesterol and sphingolipids, also known as lipid rafts (Simons and Ikonen, 1997) or the related structures called caveolae (Anderson, 1998). The reported extent of Ras association with these domains varies between studies, often depending on whether detergent has been used in their preparation (Brown and London, 1998). A plausible explanation for this variability is that the farnesyl group provides a weaker affinity to rafts than does a saturated long-chain acyl group and that this interaction is partially disrupted by detergent treatment. Recently, Hancock, Parton, and coworkers have shown that disruption of rafts has different effects on the ability of activated, oncogenic H- and K-Ras4B proteins to activate Raf1 (Roy et al., 1999). Expression of a dominant-negative version of caveolin, the principal structural protein of caveolae, or extraction of cholesterol from rafts with cyclodextrin, blocked H-Ras- but not K-Ras4B activation of Raf1. Expression of the dominant-negative caveolin displaces H-Ras from rafts/caveolae but has little effect on K-Ras4B. Intriguingly, disruption of rafts/caveolae does not interfere with the ability of H-Ras to recruit Raf1 to the plasma membrane but appears to affect Raf1 activation. This suggests that it is association with rafts/caveolae rather than the bulk plasma membrane that is required for activation of Raf1. H-Ras and K-Ras4B may associate with physically different rafts/caveolae that are differentially dependent on cholesterol, or the raft/caveolae association of H-Ras may be more sensitive to cholesterol content than that of K-Ras4B. This could be due to the use of farnesylation/S-acylation in the case of H-Ras, where insertion of the lipids into the bilayer is the predominant driving force for membrane association, compared to the farnesylation/polybasic combination of K-Ras4B, where a substantial amount of membrane binding energy comes from bilayer-independent electrostatic forces.

To make things a little more complicated, disruption of caveolin function in the nematode worm *C. elegans* leads to a phenotype that is consistent with hyperactivation of the Ras/Raf/MAP kinase pathway (Scheel et al., 1999). It is not clear why disruption of rafts/caveolae should lead to hyperactivation of the MAP kinase pathway. However, a difference between the observations with *C. elegans* compared to those with mammalian cells is that the worm system depends on the activation of normal Ras and subsequent signaling whereas in the mammalian cell experiments oncogenic activated Ras is being expressed. The single Ras protein in *C. elegans* appears to be more like K-Ras4B than N/H-Ras because it has a polybasic domain. Perhaps localization of K-Ras4B-like isoforms to rafts/caveolae suppresses their activation whereas N/H-Ras-like isoforms of Ras require localization to rafts to activate signaling pathways.

Interference with the posttranslational modifications of Ras has the potential to be an important therapeutic strategy for cancer. This study from Choy et al. (1999) on the trafficking of Ras to the plasma membrane together with the increasing interest in lipid subdomains and signal transduction suggests that investigation of the membrane association of Ras will continue to be a fruitful area of research.

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